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1 Preface

In the integrated NORMAN documents V.1.1a, V2.1 and V3.1 on validation of chemical and biological methods, guidelines have been formulated to help the user (i.e. researcher, regulatory agency, etc) validate and improve his/her analytical method for use at either research level, expert level or routine level (Schwesig, 2007).

However, for the first identification of an unknown compound occurring in an environmental compartment that could be regarded as an emerging pollutant, logically no targeted analytical method is available. For instance, in the majority of the European aquatic ecosystems, including river basins as well as coastal zones, monitoring programmes are run in order to assess their quality in terms of contamination by (toxic) chemicals. Emerging pollutants are generally not included in such programmes due to the lack of awareness of their occurrence and possibly even of their toxicological properties. However, in order to protect the environment as well as the European society as a whole, it is crucial to assess whether there are as yet unknown compounds occurring in the environment that may pose a significant health risk.

Discovery of an emerging pollutant may occur either by chance or through a dedicated survey when a specific chemical is expected to occur in the environment. Yet another possibility is to find and identify emerging pollutants through the combined use of chemical and biological methods: in case a biological response is observed in a certain test method, chemical analytical techniques are applied to identify to which compound this can be attributed. This approach is called Effect Directed Analysis (EDA) or Toxicity Identity Evaluation (TIE). The latter term describes standardized methods that have been developed by the US-EPA for effluent quality assessment and classification of contaminant groups on a more or less routine basis (Norberg et al., 1991, Mount et al, 1989, Mount, 1989). The EPA TIE is a stepwise approach and uses the responses of the bioassays to detect which toxicant groups are present in a sample; for an overview see Fig 1. The strategy is based on the idea to reduce the number of possible candidates that are responsible for the toxic effect. In the first phase the characterisation of the toxicants (e.g. solubility, volatility, metals, etc) in effluent will be established (phase I) (Fig. 2). This phase will test the physical/chemical properties of the compound of interest. The bioassay tests are performed before and after several treatment steps, such as filtration or purging, to provide information on the character of the compounds. The result of this exercise is that toxicants can be classified according to their predominant characteristics, e.g. in cationic metals, non-polar organics, oxidants, pH related toxicity, volatile, etc. In the second phase the identity of the toxicant should be confirmed. Depending on the classification, several methods are available for this (Mount et al., 1989). If the toxicant is identified, the final phase (phase III) is to confirm that the identified toxicant is responsible for the observed toxic effect.
In this document the focus is on EDA, which refers to the integrated use of biological and chemical test methods in a broader context, for which no standardized guidelines are available.

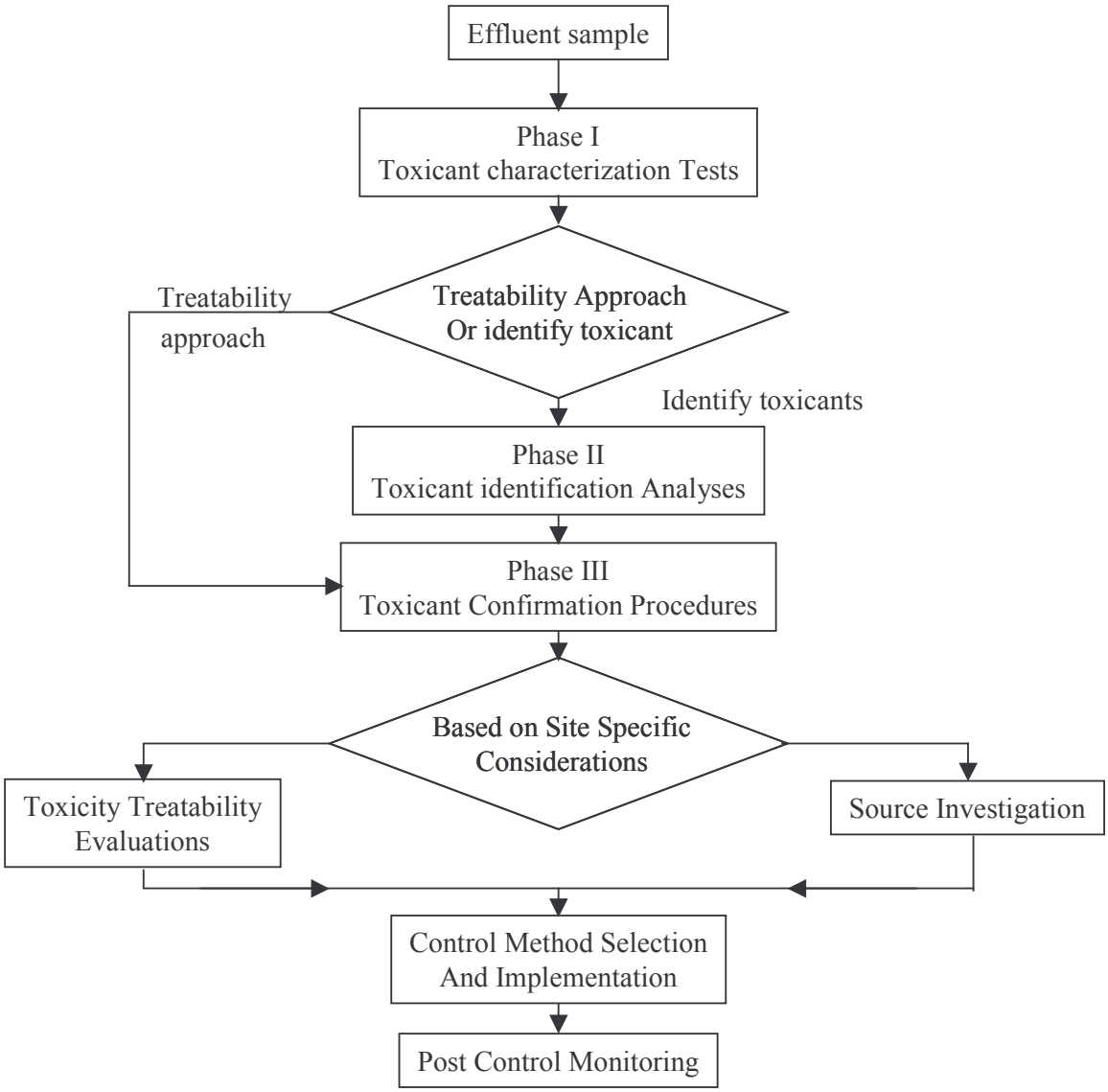

Fig 1: Flow chart of toxicity reduction evaluations including the identification of the toxicants that are responsible for the toxic effect, according to Norberg et al., 1991.

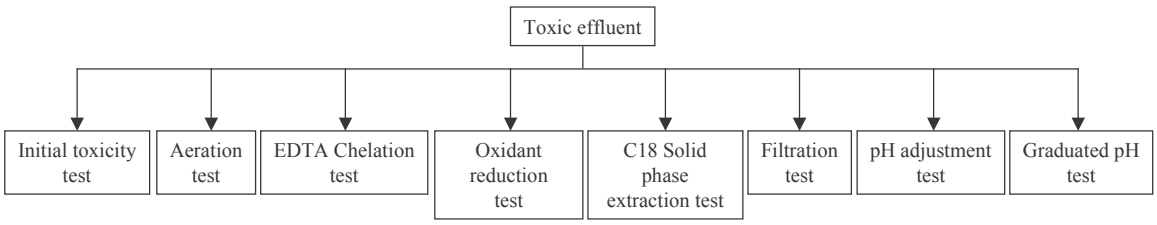

Fig 2: Overview of effluent toxicity characterisation test according to TIE EPA protocol (Norberg et al., 1991). After each treatment the remaining extract is tested with bioassays.

2 Aims & Scope

For the discovery of emerging pollutants through EDA - indicating the integrated use of chemical and biological techniques, this protocol will provide the reader with a set of recommendations for the various issues to be addressed:

- Sample preparation and cleanup
- Selection of in vitro/in vivo bioassays to be used
- Selection of fractionation procedures
- Identification of compounds responsible for biological response
- Confirmation of toxicity of the identified compounds

This protocol covers a broad range of quantitative and qualitative biological and chemical test methods for EDA of water (including inland and marine water, ground water and waste water) and soil (including sediment). EDA approaches for environmental compartments like biota and air have been only sparsely, if at all, reported in the scientific literature and will therefore not be addressed here.

To fully benefit from the potential of an EDA approach to trace and identify unknown, emerging pollutants, a tailor-made strategy should be designed that includes the compilation of existing monitoring data (if any) and knowledge on inputs into the ecosystem, such as industrial areas, the presence of sewage treatment plants, etc. This will facilitate the choice of sampling locations for a full EDA as well as the type of assays to implement.

Clearly, the different validation levels as described in the integrated NORMAN Validation protocol (Schwesig, 2007) still hold for the separate steps included in an EDA, e.g., the in vitro/in vivo assays and chemical analysis. Referral should be made to that protocol for validation purposes. The current protocol describes the EDA strategy that includes the integration of chemical and biological techniques and can be used in addition to the aforementioned NORMAN Validation protocol.

3 Effect-directed analysis (EDA)

3.1 General approach

EDA is a combination of extraction, biological testing, fractionation, and chemical analysis with the main aim to reduce the complexity of the mixture of compounds in order to enable the identification of the toxicant(s) responsible for the observed biological response. EDA has mainly been developed for the identification of organic toxicants in complex samples with best results obtained for compounds with specific mode of actions. Metals and inorganic compounds are usually excluded in this approach, because they are easy and cheap to measure.

In general, an EDA starts with the extraction/clean-up of the sample, and this so-called whole extract is tested with bioassays to establish the biological response. If the extract does not exhibit biological activity in the selected bioassay, no further action is needed. Responsive extracts are fractionated using a variety of fractionation techniques in order to reduce the number compounds in a specific fraction and therefore the number of possible candidates responsible for the biological activity. The fractions are tested again in the selected bioassay(s) to find the fraction(s) that are biologically active. If necessary, further fractionation and biotesting can be performed to reduce the complexity of the mixture even further, with the final goal to have a few individual compounds left in the biologically active fraction(s). The next step is to identify and quantify the compounds in the active fraction. The final step is to confirm that the identified compound is indeed responsible for the observed activity.

The full EDA process is shown in Figure 3. A recent review by Brack (2003) gives an excellent overview of the possibilities and limitations of EDA for the identification of organic toxicants in the environment. The next sections are based on this review and a number of selected EDA studies.

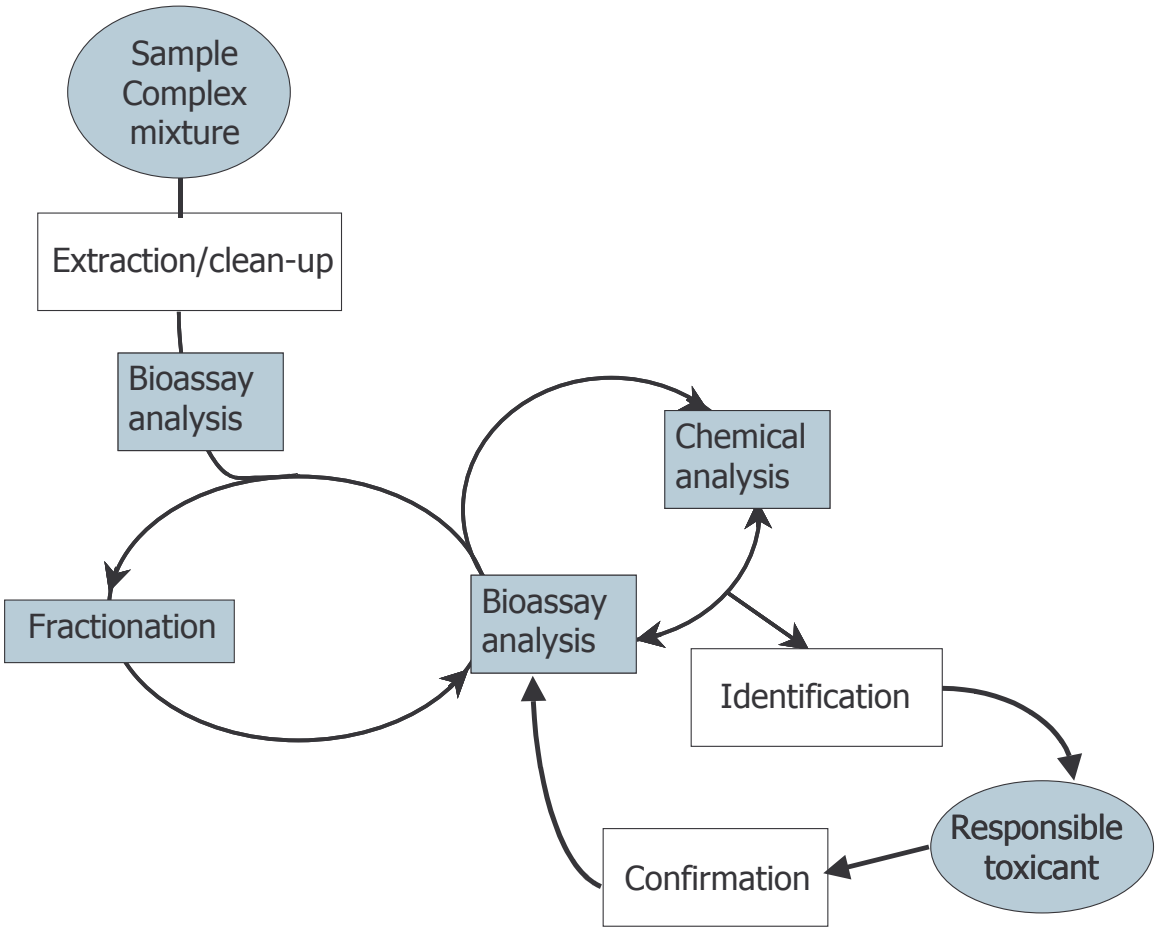

Figure 3: The concept of effect-directed analysis (EDA). Diagram based on Brack (2003).

3.2 Sample preparation and cleanup

The first step of an EDA is the extraction of the sample, as water, sediment, soil, or air particles cannot be tested directly by in vitro bioassays. With in vivo assays the extraction step is not always necessary, and direct testing is a possibility. Validation of the extraction method can be performed according to the NORMAN Validation protocol for research methods (Schwesig, 2007). It should be stressed that, differently from the analysis of target compounds, EDA requires the extraction of a broad range of compounds that have widely differing physical-chemical properties, as it is unknown beforehand which toxicant is causing the biological effect. The consequence is that a compromise has to be made with regard to the most suitable extraction solvent. In target analysis the solvent with the highest extraction efficiency is normally selected, in EDA often a mixture of solvents is used to be able to extract both hydrophobic as well as hydrophilic compounds. This observably results in extraction efficiencies that are depending on the class of compounds and can easily vary between 30 and 100%. As an example, a mixture of dichloromethane:acetone was able to extract 17 compounds from sediment, that had large differences in physical–chemical properties, with recoveries between 60 and 120%, the average recovery was 86% (Houtman et al., 2007).

For water samples occasionally sequential extractions are used to extract neutral, basic, acidic, hydrophobic, and hydrophilic compounds (Reemtsma et al. 1999; Fiehn et al., 1996, 1997). In the past, EDA extraction methods for water focussed on lipophilic compounds (e.g.

PAHs, PCBs), but in recent years more and more attention was paid to polar and ionic compounds (Amato et al., 1992; Jop et al., 1991; Huckins et al. 1990).

An important step after extraction of the sample, especially for sediment, soil, and biota, is the removal of matrix compounds (e.g. sulphur, humic acids, lipids) previous to biotesting. Matrix compounds can interfere with the bioassay, can cause cytotoxicity or can mask the biological response. In EDA the clean-up method is frequently a compromise between removal of matrix compounds and collection of the compounds of interest that exhibit a broad range of physical-chemical properties (e.g. Houtman et al., 2007). Validation of the clean-up step can be performed according to the NORMAN Validation protocol (Schwesig et al., 2007), but should be evaluated both chemically (e.g. recoveries) and biologically (e.g. background activities of solvents, cytotoxicity).

An aspect of EDA that differs from the development of an extraction method for target analysis is that the purity of extraction solvents should be tested both chemically as well as biologically in the selected bioassay(s). Even high quality grade solvents that are suitable for target analysis, and contain no impurities or compounds that chromatographically coelute with the target compound, may contain compounds such as stabilizers or preservatives that are toxic or elevate the activity observed in the bioassays.

Another aspect of the integrated use of chemical and biological methods is the change of solvent that is needed for the application of the organic extract in a bioassay carried out in an aqueous medium. The organic extraction solvent used for the extraction of the sample is often immiscible with water, and a transfer step is needed from the extraction to a solvent that does mix with water. During this transfer step compounds may precipitate as they may have different solubility characteristics in the new solvent, leading to a reduced availability of the compounds in the biological test system. In addition, evaporation of relatively volatile compounds in the original extract may occur due to the solvent change procedure, which often includes an evaporation step. Validation of the solvent exchange step is necessary.

3.3 Selection of in vitro/in vivo bioassays to be used

For biotesting the whole extract and subsequently also the fractions, many bioassays can be used in EDA studies; however, not all are ideal and sometimes a compromise is unavoidable. An overview of in vitro and in vivo bioassays used in TIE/EDA approaches can be found in a recent SETAC publication (Norberg-King et al., 2005) and in the framework of the EU Integrated Project Modelkey (Thomas et al., 2006). Some of the bioassays are adequately validated and QA/QC criteria have been formulated. The following six criteria are guidelines for selection of bioassays for EDA studies:

- Small scale bioassays
- Sensitivity
- Speed
- High-throughput
- Cost-effectiveness
- Validation

Small scale assays are recommended for reasons of maximum throughput, as many samples/fractions have to be run in EDA studies. Sensitivity is another aspect that is highly important, because compounds with a high biological activity may be present at very low concentrations. Generally, the activity is reduced after fractionation as active compounds may be distributed in different fractions, and therefore, the sensitivity of the bioassay is essential. In vitro assays with specific end-points such as Ah-receptor, estrogen or androgen receptor binding are more powerful and have been successfully applied in EDA studies (e.g. Dobiás et al., 1999; Houtman et al., 2006a, 2006b; Marvin et al., 2000, 2006; Nishioka et al., 1988; Thomas et al., 1999; 2001, 2002).

3.4 Selection of fractionation procedures

After the first biotesting, the next step for biologically active extracts is their fractionation. Fractionation is the most important step in EDA to reduce the complexity of the initial mixture of compounds in the whole extract. The fractionation provides general information on the properties of the compounds, depending on the chosen fractionation technique and mechanism. Possible fractionation mechanisms can be based on hydrophobicity, molecular size, planarity etc. The choice of fractionation phase is partly governed by the expected presence of certain classes of compounds in the extract. Combinations of different chromatographic fractionation techniques such as Normal Phase and Reversed Phase liquid chromatography are often necessary to reduce the complexity of the extract to a few individual compounds. Validation of the different fractionation techniques can be achieved by implementation of the NORMAN Validation protocol for either research, expert or routine level. The above-mentioned issues of solvent purity and solvent change are also applicable for the fractionation method.

All thus obtained fractions should be tested with the bioassay(s) to identify the active or responsive fractions. Often further fractionation, using a different type of chromatographic separation, is needed to reduce the complexity of the active fractions even further in order to facilitate identification of individual compounds in the responsive fractions.

3.5 Identification of compounds responsible for biological response

After fractionation and biotesting to select the active fractions, chemical analysis is applied for identification of compounds. Mass spectrometric detection is often the method of choice. Firstly, broad screening techniques such as GC-MS full scan analysis, are used as a first attempt to identify the compounds in the active fractions. However, in many cases additional techniques that have higher chromatographic resolving power (e.g. comprehensive GCxGC approaches) in combination with higher mass accuracy (e.g. Time-of-Flight MS) are required to obtain the necessary information for eventual identification of the active compounds.
The use of GC-based screening and identification techniques facilitates the searching in well established libraries such as NIST, although it should be kept in mind that there are still great numbers of chemicals not included in any searchable library. The predominant use of GC-based techniques for chemical analysis in EDA intrinsically means that identification of unknown, emerging pollutants that are polar, have limited volatility as well as limited thermostability, is seriously compromised. To date, the application of LC-MS techniques for structure elucidation and identification of unknowns in environmental matrices has been negligible.
For identification purposes, quantification of the active compounds is of secondary importance, however, knowledge on the concentration of active compounds is needed for the confirmation of biological effects.

3.6 Confirmation

In EDA studies, confirmation of the findings deals with i) analytical chemical and ii) biological activity confirmation. For both aspects, the presence of neat standards is of major importance, but in practice this is a serious problem, however trivial.
Confirmation of the chemical identification of a compound is a challenging task: a clear yes/no answer is rarely obtained. In most cases, a stepwise approach that leads to an increase of evidence of the presence of a specific compound is followed through the collection data based on GC retention times, mass, NMR, UV and IR spectra in combination with capacity factors on different LC columns.

Confirmation of the observed effect caused by the tentatively identified compound in the selected bioassay should provide evidence that indeed the compound is responsible for the effect. In EDA almost all confirmation studies rely on the concept of Concentration Addition (CA), which is valid for compounds with a similar mode of action in a given bioassay.

Responses are usually expressed as effect specific Equivalent Quantities (EQs), such as Estrogenic Equivalent Quantities (EEQs) for the ER-CALUX assay (see also Houtman et al., 2006b). In the case of dissimilar modes of action, the observed effect is accounted for through the concept of Independent Action (IA). If possible, biological effect confirmation should provide a full dose-response curve and not just an EC50 value, because major mistakes are easily made upon extrapolation of these data.

At present, confirmation in EDA is an evolving concept that is being developed and evaluated within the EU project Modelkey by Brack et al. When applying EDA, attention should be paid to the progress in this field of research.

4 Overview validation parameters

In conclusion, the following qualitative aspects should be considered for validation of an EDA. The basic validation requirements for either a chemical or a biological method have been provided in the NORMAN Validation protocol document V1.1a (Schwesig, 2007).

Table 1: Additional requirements for the validation steps in an EDA. Basic requirements can be found in the NORMAN Validation protocol (V1.1a, Schwesig, 2007).

Procedure	**Requirements**
Sample preparation and cleanup	
	Extraction efficiency based on broad range of compounds, compromise often needed
	Clean-up step to remove matrix compounds and collection of a broad range of compounds of interest, compromise often needed
	Change to solvent suitable for biotesting
	Purity of extraction solvent
In vitro/in vivo bioassays	
	• Small scale bioassays • Sensitivity • Speed • High-throughput • Cost-effectiveness • Validation according to Validation protocol V1.1a
Fractionation	Validation of method according to Validation protocol V1.1a
Identification of compounds responsible for biological response	High resolving power of the separation technique in combination with high mass accuracy in detection
Confirmation	Availability of neat standards
	Combination of all chemical analytical data, e.g. mass, NRM, UV, IR spectra; capacity factors and GC retention times
	Full dose response curve in bioassay

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